IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Millar, et al.
Appl. No.	:	10/543,017
Filed	:	July 24, 2006
For	:	ASSAY FOR DETECTING METHYLATION CHANGES IN NUCLEIC ACIDS USING AN INTERCALATING NUCLEIC ACID
Examiner	:	Chunduru, S.
Group Art Unit	:	1637

DECLARATION UNDER 37 CFR §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Dr. Douglas Spencer Millar, declare and state:

1. I am an inventor of the above-referenced application, and am a Senior Principal Research Scientist at Human Genetic Signatures Pty Ltd., the assignee of the above-referenced patent application (***the Application***).

2. I am an expert in the field of molecular biology including DNA methylation. My Curriculum Vitae is enclosed (Exhibit A).

3. I am a designated inventor on 18 patents or pending patent applications, I have authored 20 peer reviewed scientific papers in the field of molecular biology and sodium bisulfite conversion of DNA, including the original 1992 PNAS paper that first described the bisulphite sequencing protocol, and have presented at 14 scientific meetings (with a further 3 scheduled in Florida, Barcelona and Boston for 2008)

4. My research currently relates to the use of sodium bisulphite conversion of DNA to aid in the measurement of methylation in nucleic acids, the detection of microorganisms, and to the use of intercalating nucleic acids in such methods.

5. I am familiar with the Application and pending claims. I understand that claims 33-44, 46, and 55-59 were rejected as obvious over Eads et al. (*Nucleic Acids Res.* 28:e32 i-viii, 2000) in view of Christensen et al. (*Nucleic Acids Res.*, 30:4918-4925, 2002), and that claims 45 and 47-54 were rejected as obvious over this combination of references, further in view of Shah et al. (US 5,629,156). I have reviewed the pending claims and these references, and herein describe why the intercalating nucleic acids (INA) described by Christensen et al. would not work in the method described by Eads et al.

6. The assay described by Eads et al. uses conventional oligonucleotides, PRC primers and a specific oligonucleotide labeled with reporter and quencher groups to detect the presence of specific target molecules. The method of Eads et al. relies on amplification of the target molecule by PCR to produce enough molecules to be detected by real time TaqMan labeled probes.

7. Prior to the present invention, INAs had not been used in any type of amplification reaction using either conventional or bisulphite-treated DNA as a template.

8. From the teaching of Christensen et al, INA primers would not be expected to work if used in the amplification reaction of Eads et al since the amplification enzyme (Taq polymerase) would not be expected to extend past the INA due to the presence of the intercalator pseudonucleotide (IPN) group which would be expected to hinder primer extension.

9. In fact, my laboratory has conducted experiments to assess the ability of Taq polymerase to extent INAs. We found that when an IPN or multiple IPNs were placed near or at the 3' end of an INA, the IPN blocked extension by Taq resulting in no amplification of the desired target sequence. Thus, INAs would not be expected to work properly in a TaqMan assay (as disclosed by Eads et al.), since their inherent nuclease resistant properties (as disclosed by Christensen et al), would be expected to prevent probe hydrolysis, thus stopping the reaction.

10. When using bisulfite treated DNA the Tm of the primers are significantly lower that the equivalent Tm of a primers using wild type, untreated DNA. This can result in the amplification of artefacts due to the low temperatures required in the annealing step of the amplification or low stringency hybridization steps which can cause false positive amplification/hybridization signals, which is a significant problem when using bisulfite treated samples and can result in having to perform two round nested PCR reactions to improve specificity, or to use internal probes as was done by Eads et al. This property of bisulfite treated DNA can also severely reduce the specificity in hybridization based approaches using conventional oligonucleotides. We have found that one major advantage of INAs for bisulfite methylation work is that INAs can increase the Tm at which the amplification or hybridization reactions can be done using bisulfite treated DNA as a template to improve specificity, which is neither disclosed nor suggested by Eads et al or Christensen et al.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

By: _____ Date: 12/03/08

Dr. Douglas Spencer Millar

CURRICULUM VITAE

Name: Douglas Spencer Millar

Tertiary Qualifications

1990-1995: Ph.D. Molecular Microbiology, St George's Hospital Medical School, Department of Surgery, University of London. Ph.D. project: *Mycobacterium paratuberculosis*, mycobacteria and chronic enteritis in humans and animals.

1986-1987: Postgraduate training, Strathclyde University, Immunology Division, Department of Bioscience and Biotechnology, 31 Taylor Street, Glasgow.

1982-1986: B.Sc. (Hons. 2.1) in Applied Microbiology, Caledonian University, Cowcaddens Road, Glasgow, G4 OBA, Scotland. Honours Project: Bacterial populations associated with *Lemna minor*.

Research and Work Experience

2001-Present Human Genetic Signatures Riverside Corporate Park, Level 4, 11 Julius Avenue, Sydney, NSW 2113. Chief Research Scientist:

1995-2001: CSIRO Division of Molecular Science, 2 Richardson Place, Delhi Road, Sydney, NSW 2113. **Senior Research Officer:**

1990-1995: Ph.D., University of London. St George's Hospital Medical School, Department of Surgery, University of London.

Jan-July 1990: Kanematsu Laboratories, RPAH, Camperdown, NSW 2050. **Research Assistant.**

1987-1989: Wellcome Diagnostics, Langley Court, Beckenham, Kent, BR3 3BS
Senior Technician, Hepatitis and new technologies section.

Publications

Frommer M, Mcdonald C, **Millar DS** *et al* (1992). 5-Methylcytosine in the kininogen gene promoter revealed by a positive strand-specific reaction. *Proc.Nat.Acad.Sci.*, USA **89**: 1827-1831.

Millar DS, Withey SJ, Tizard MLV, Ford JG, Hermon-Taylor J (1995). Solid phase Hybridisation Capture of low abundance target DNA sequences: Application to the PCR detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp.*silvaticum*. *Anal.Biochem.* **226**: 325-330.

Millar DS, Ford JG, Sanderson J, Withey SJ, Tizard JLV, Doran T and Hermon-Taylor J. (1996). IS*900* polymerase chain reaction for *M.paratuberculosis* applied to retail supplies of whole pasteurised cow's milk in England and Wales. *Applied and Environmental Microbiology*. **62**(9): 3446-3452.

Doran TJ, Tizard MLV, **Millar DS**, Ford J, Sumar N, Loughlin M and Hermon-Taylor J (1997). IS*900* targets translation initiation signals in *Mycobacterium avium* subsp. *paratuberculosis* to facilitate the expression of its *hed* gene. *Microbiology* **143**: 547-552.

Strizaker, C, **Millar DS**, Paul CL, Warnecke PM, Harrison J, Dryja TP, Vincent PC, Frommer M and Clark SJ (1997). Extensive DNA methylation spanning the RB promoter in retinoblastoma Tumours. *Canc. Res.* **57**: 2229-2237.

Warnecke PM, Strizaker C, Melki JR, **Millar DS**, Paul CL and Clark SJ (1997). Overcoming PCR bias in quantitative methylation analysis of bisulphite-treated DNA. *Nuc. Acids Res.* **25**: 4422-4426.

Mark Tizard, Tim Bull, **Douglas Millar**, Tim Doran, Helene Martin, Jon Ford and John Hermon-Taylor (1998). A low G+C content element in *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* subsp. *silvaticum* with homologous genes in *M. tuberculosis*. *Microbiology*. **144** 3413-3423.

Millar, D. S., Ow, K., Paul, C. P., Russell P. J., Molloy, P.L., and Clark, S.J. (1999). Detailed methylation analysis of the Gluthatione S-transferase (GST-P1) gene in prostate cancer. *Oncogene*. **18**(6): 1313-24.

Molloy, P.L., **Douglas Millar**, Pamela Russell and Susan Clark. Prostate Cancer DNA Methylation Assay. *Today's Life Sciences* (October 1999).

Marsh I, Whittington R, **Millar D** (2000) Quality control and optimized procedure of hybridization capture-PCR for the identification of mycobacterium avium subsp. paratuberculosis in faeces. *Mol Cell Probes*. Aug;14(4):219-32.

Jackson P, **Millar D**, Kingsley E, Yardley G, Ow K, Clark S, Russell PJ. (2000) Methylation of a CpG island within the promoter region of the KAI1 metastasis suppressor gene is not responsible for down-regulation of KAI1 expression in invasive cancers or cancer cell lines. *Cancer Lett*; 157(2):169-76.

Millar DS, Paul CL, Molloy PL, Clark SJ. (2000). A distinct sequence (ATAAA)n separates methylated and unmethylated domains at the 5'-end of the GSTP1 CpG island. *J Biol Chem*; 275(32):24893-9.

Paul Jackson, **Douglas Millar**, Elizabeth Kingsley, Gina Yardley, Kim Ow, Susan Clark, Pamela J Russell (2000). Methylation of a CpG island within the KAI1 promoter region is not responsible for reduced KAI1 mRNA expression in invasive bladder cancer cell lines. *Cancer Letters* **157**: 169-176.

Marsh, I., Whittington R and **Douglas Millar** (2000). Quality control and optimised procedure of hybridisation capture-PCR, for the identification of *Mycobacterium avium* subsp. *paratuberculosis* in faeces. *Mol. Cell Probes* **14**(4): 219-32.

Welch J, **Millar D**, Goldman A, Heenan P, Stark M, Eldon M, Clark S, Martin NG, Hayward NK (2001). Lack of genetic and epigenetic changes in CDKN2A in melanocytic nevi. *J Invest Dermatol.* Aug;**117**(2):383-4.

Chetcuti A, Margan SH, Russell P, Mann S, **Millar DS**, Clark SJ, Rogers J, Handelsman DJ, Dong Q (2001). Loss of annexin II heavy and light chains in prostate cancer and its precursors. *Cancer Res.* Sep 1;**61**(17):6331-4.

Schmitt JF, **Millar DS**, Pedersen JS, Clark SL, Venter DJ, Frydenberg M, Molloy PL, Risbridger GP (2002). Hypermethylation of the inhibin alpha-subunit gene in prostate carcinoma. *Mol Endocrinol.* Feb;**16**(2):213-20.

Millar DS, Warnecke PM, Melki JR, Clark SJ. (2002) Methylation sequencing from limiting DNA: embryonic, fixed, and microdissected cells. *Methods.* Jun;27(2):108-13.

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Methods Mol Med.;81:219-40.

Cristina Baleriola, **Douglas Millar**, John Melki, Neralie Coulston, Phillip Altman, Nikolas Rismanto and William Rawlinson. (2008) Comparison of a novel HPV test with the Hybrid Capture II (hcII) and a reference PCR method shows high specificity and positive predictive value for 13 high-risk human papillomavirus infections. In Press *J. Clin. Virol.* Feb 7

Book Chapters

Douglas S Millar, Robin Holliday and Geoffrey W Grigg (2003). History and significance of the 5th base. in; The Epigenome, S Beck and A Olek (eds), 1-18, Wiley.

Hermon-Taylor J, Tizard ML, Sanderson J, Kempsell K, Sumar N, **Millar DS**, Loughlin M, Ford J, Withey S. (1994) Mycobacteria and the etiology of Crohn's disease. In: Rachmilewitz D (ed). Inflammatory Bowel Diseases. London: Kluwer Academic Publishers, 51-57.

Patents

Novel polynucleotides and polypeptides in pathogenic mycobacteria and their use as diagnostics, vaccines and targets for chemotherapy. Great Britain Patent number PCT/GB96/03221, filed June 19 1998. Inventors John Hermon-Taylor, Mark Tizard, Mark Loughlin, Nazira Sumar, John Ford, Tim Doran and **Douglas Millar**.

Diagnostic Assay (Detection of methylated GST-P1 gene in prostate cancer). Australian Provisional application PP3129, filed April 1998. Inventors **DS Millar**, PL Molloy & SJ Clark.

Sandwich PNA Assay for detection of nucleic acid sequences. Australian Provisional application. filed September 2000. Inventors **DS Millar**, PL Molloy & Griggs GW (2000).

Restoration of Methylation States in Cells. PCT Application No PCT/AU2003/001573
Priority Date 26 November 2002. International Filing Date 26 November 2003
Inventors **Douglas Millar**, John Melki, Geoffrey Grigg, George Miklos

Treatment of Methylated Nucleic Acid. US Patent Application No 10/428310
Filing Date 2 May 2003. PCT Application No PCT/AU2004/000549
Priority Date 2 May 2003. International Filing Date 29 April 2004
Inventors **Douglas Millar**, Cassandra Vockler, Keralie Coulston.

Methods for Genome Amplification. PCT Application No PCT/AU2004/000722
Priority Date 17 June 2003. International Filing Date 31 May 2004. Inventors **Douglas Millar**

Nucleic Acid Detection Assay. PCT Application No PCT/AU2004/0001196

Priority Date 4 September 2003. International Filing Date 3 September 2004
Inventors **Douglas Millar**, John Melki, George Miklos.

Assay for Detecting Methylation Changes in Nucleic Acids using an Intercalating Nucleic Acid. PCT Application No PCT/AU2004/000083. Priority Date 24 January 2004 International Filing Date 23 January 2004. Inventors **Douglas Millar**, John Melki, Geoffrey Grigg, George Miklos

Amplification Blocker. PCT Application No PCT/AU2005/001374
Priority Date 10 September 2004. International Filing Date 9 September 2005
Inventor **Douglas Millar.**

Detection of Microorganisms. AU Provisional Application No 2004906915
Filing Date 3 December 2004. Completion Date 3 December 2005.
Inventor **Douglas Millar.**

Detection of Human Papilloma Virus. US Provisional Application No 60/638625
Filing Date 23 December 2004. Completion Date 23 December 2005
Inventors **Douglas Millar**, George Miklos, John Melki.

Isothermal Amplification. US Provisional Application No 60/685697
Filing Date 26 May 2005. Completion Date 26 May 2006
Inventors **Douglas Millar**, Geoffrey Grigg.

Assay for a Health State. US Provisional Application No 60/717148
Filing Date 14 September 2005. Completion Date 14 September 2006
Inventors **Douglas Millar**, John Melki

Vector carrying a polynucleotide which encodes a polypeptide having the ability to stimulate an immune response against the polypeptide of Seq ID No:24
Publication number: US2006204521. Publication date: 2006-09-14
Inventors: Hermon-Taylor John (GB); Doran Tim (AU); **Millar Douglas** (AU); Tizard Mark (GB); Loughlin Mark (GB); Sumar Nazira (GB); Ford John (GB)

Modified microbial nucleic acid. PCT Application No PCT/AU2006/000755 Filed 2 June 2006
Inventors **Douglas Millar**, John Melki

Assay for Gene Expression. AU Provisional Application No 2007901397 Filed 16th March 2007
Inventors **Douglas Millar**, Geoffrey Grigg

Enzymes for Amplification and Copying Nucleic Acids. Filed 30th October 2007
Inventors **Douglas Millar**, Geoffrey Grigg

Oral Presentations

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"IS*900* polymerase chain reaction for *M.paratuberculosis* applied to retail supplies of whole pasteurised cows milk in England and Wales."

Doug Millar. CSIRO Molecular Science, Sydney. Nov. 1995. "*Mycobacterium paratuberculosis* and chronic enteritis in humans and animals.

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Doug Millar, Peter Molloy, Pam Russell and Susan Clark. International Hormone Responsive Cancers Meeting, Adelaide in Feb, 1997. "Analysis of methylation patterns of the GST-π gene promoter in Prostate Cancer".

Doug Millar, Peter Molloy , Pam Russell and Susan Clark 1998. Wilhelm Symposium on Prostate Cancer, Australasian Society For Experimental Pathology, University of Sydney, October 1st, 1998. "DNA methylation and Prostate cancer."

Doug Millar, Peter Molloy and Susan Clark. Johnston & Johnston Research Laboratories, Rushcutters Bay, Sydney, March 1999. "Methylation and Prostate Cancer-MSP PCR as a new diagnostic tool for the early detection of cancer".

Douglas S Millar, John R Melki and Geoffrey W Grigg. RNA Methylation. Epigenetics Australian Scientific Conference, 2007 Perth, 4 - 7 November

Poster Presentations

Withey S, **Millar DS**, Tizard M et al (1993). Analysis of DNA extracts of normal and diseased intestine using PCR for the 32kDa general Mycobacterial antigen. United European Gastroenterology week, Barcelona 1993.

Millar DS, Withey SJ, Tizard MLV, Ford JG, Hermon-Taylor J. Solid phase Hybridisation Capture of low abundance target DNA sequences: Application to the PCR detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp.*silvaticum* 1994. 4th International Colloquium on Paratuberculosis. Cambridge, UK.

D. Millar, C. Paul, C. Stirzaker and S.J. Clark 1996. DNA Methylation Of The Retinoblastoma Gene. *The Organisation and Expression of the Genome, 18th Annual Genome Conference,* Lorne, Vic.

Clare Stirzaker, **Doug Millar** and Susan Clark. FASEB conference on biological methylation in Vermont, USA, in June 14-19, 1997. "Extensive DNA Methylation Spanning The Rb Promoter In Retinoblastoma Tumors".

Susan Clark, Clare Stirzaker and **Doug Millar**. FASEB conference on biological methylation in Vermont, USA, in June 14-19, 1997."DNA Methylation in Tumour Suppressor Genes Provides a Clue".

Doug Millar, Peter Molloy and Susan Clark Inaugural National Meeting of the Genito Urinary Oncology Group in Leura, NSW in July 25-27 1997. "Extensive Methylation Of GST-Pi in Prostate Cancer"

Doug Millar, Peter Molloy and Susan Clark Inaugural Marie Curie workshop on DNA methylation and Epigenetics. Paris, France. November 1998. " Methylation Of GST-Pi gene in Prostate Cancer".

D. S. Millar, C. J. Vockler, J. R. Melki. The Application of a Novel DNA Simplification

Approach for the Universal Detection and Subtyping of Gram Positive Bacteria. 2007, ECCMID Munich, 31 March to 3 April

N.A. Coulston, S.P. Siah, **D.S. Millar**. Specific Detection of High-Risk HPV Genotypes Using a Novel DNA Simplification Strategy. American Society for Microbiology, 2007, Toronto, 21-25 May

Cassandra Vockler and **Douglas Millar**. Methylation profiling of embryonic and adult stem cells. 2007, ISSCR, Cairns, 17-20 June.

Shoo Peng Siah and **Douglas Millar**. Detection of Human Papillomavirus in archival tissue sample. Australian Society for Microbiology, 2007, Adelaide, 9-13 July 2007

N. Boulter, S.P. Siah, C.J. Vockler, N.A. Coulston, K. Warton, P. Rajasekariah, J.R. Melki, **D.S. Millar** (Sydney, AU). Specific detection of hepatitis C virus in clinical samples using a novel simplification strategy.ECCMID Barcelona 19 - 22 April 2008.

N. Boulter, S-P. Siah, C. J. Vockler, N. A. Coulston, K. Warton, P. Rajasekariah, J. R. Melki, **D. S. Millar**, Optimization of a Novel RNA Simplification Strategy for the Sensitive Detection of Hepatitis C in Clinical Samples. ASM Boston, 108th General Meeting, June 1- June 5, 2008.

N. Boulter, S. P. Siah, C. J. Vockler, N. A. Coulston, K. Warton, P. Rajasekariah, J.R Melki and **D. S. Millar.** A Novel Nucleic Acid Simplification Procedure Capable of the Detection Multiple Strains of both DNA and RNA Viruses in a Single Tube. 24th Annual Clinical Virology Symposium and Annual Meeting of the Pan American Society for Clinical Virology, Daytona Beach, FL. April 27 - 30, 2008.

Prizes and Awards

Withey S, Millar DS, Tizard M et al (1993). Analysis of DNA extracts of normal and diseased intestine using PCR for the 32kDa general Mycobacterial antigen. Prize winning poster at the United European Gastroenterology week, Barcelona 1993.